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Eurythermic Growth and Synthesis of Heat Shock Proteins of Primary Cultured Goldfish Cells

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ABSTRACT—Cells derived from the tail fin of goldfish (*Carassius auratus*) are cultured at 37°C (GTF e-2) and 27°C (GTF e-3). It is observed that the doubling times of GTF e-2 cells and that of GTF e-3 cells depend solely on the incubation temperature, irrespective of difference in the temperature at which the primary culture is started. It has been reported previously that some goldfish cell lines cultured *in vitro* for a long time grew stenothermically. The present study indicated that cells derived from the primary culture retained their ability to grow eurythermically at least in the early passages.

The relationship of protein synthesis as to incubation temperature was studied empirically. When GTF e-3 cells were exposed to 37°C, four major heat shock proteins were induced. Their molecular weights were 90 K dalton (hsp 90), 70 K dalton (hsp 70), 42 K dalton (hsp 42) and 30 K dalton (hsp 30). In GTF e-2 cells these proteins were synthesized constitutively at 37°C. The levels of synthesis of these proteins were much higher than those observed when the cells were incubated at 27°C. At 40°C, hsp 70 and hsp 30 were the dominantly synthesized proteins of GTF e-2 and e-3 cells.

INTRODUCTION

Cells derived from fish and those derived from mammals can be cultured using the same medium and the same serum, but the incubation temperature is usually different. For the cultured mammalian cells, the range of permissive growth temperature is 36–39°C with an optimal at 37°C [1]. For most fish cell lines the optimal growth temperature is 20–25°C and upper limit temperature for growth is about 30°C [2]. In general, the suitable temperature for cultured fish cells correlates with the temperature of the animals natural habitat; the cultured cells derived from cold-water fish grow most rapidly at low temperature (about 20°C), while those from some tropical fish could be kept at 37°C [2 and 3 for review]. Cell lines derived from different species of fish have different optimal growth temperatures [4–6]. The RBCF-1 cells derived from the caudal fin of the goldfish grow most rapidly at 37°C. They also can grow continuously at 20°C [6]. Recently, we isolated from RBCF-1 cells two cell clones which grow only in a narrow range of temperatures (stenothermic growth), the optimal growth temperature being 37°C and 27°C. It has been shown that cell hybrids of two stenothermic clones with different optimal growth temperature could continue to grow in a wide range of temperatures (the eurythermic growth) [7]. These results suggest that the eurythermic or stenothermic characteristics of growth of goldfish cells in culture may be modified during a prolonged *in vitro* cultivation. In this report, we investigated the growth temperature for primary cultured goldfish cells, and the effects of incubation temperature on the protein synthesis of the cells.

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MATERIALS AND METHODS

Primary culture

The tail fin about 1 cm^2 of an adult goldfish with about 5 cm body length was cut off. It was soaked in 0.4% NaClO, washed in Ca^{2+}- and Mg^{2+}-free phosphate-buffered saline (PBS(−)), and cut into pieces in trypsin-EDTA (0.1% trypsin and 0.02% EDTA in PBS(−)). The small tissue pieces were gently stirred in the presence of trypsin-EDTA at

room temperature for about 60 min. The tissue pieces and dispersed cells were collected by centrifugation and seeded into two 25 cm^2 plastic flasks (Corning Glass Works, Corning, N.Y.). Each flask was incubated at 37°C (GTF e-2) or 27°C (GTF e-3). The medium used was Leibovitz's L-15 medium (GIBCO, Grand Island, N.Y.), containing 15% fetal bovine serum (Hyclone Laboratories, Logan) and the antibiotics (50 μg/ml streptomycin and kanamycin, 60 μg/ml). A half volume of the medium was renewed every 3 days. The primary culture was harvested 18 days after the inoculation, and 1×10^6 cells were inoculated into the fresh dishes with 10 ml of the medium. Both cell lines were subcultured every 3 days. The population doubling number (PDN) was calculated from the day of first subcultivation by log (cumulative growth ratio)/log 2.

Growth curve

Cells were seeded in culture petri dishes at 2.0×10^5 cells/dish. For each cell line half the dishes were incubated at 27°C, half at 37°C. Each 3 days 3 dishes were counted for the number of cells. This was continued for 12 days. The population doubling number (PDN) of the GTF e-2 was 3.2. The PDN of the GTF e-3 was 1.3.

Protein analysis

To analyze the protein synthesis of GTF cells at various temperatures, each flask was inoculated with 5×10^5 cells (GTF e-2 at PDN 10.8, and GTF e-3 at PDN 4.2) and incubated for 10 hr at 37°C or 27°C, respectively. Before starting labeling, cells were cultured for 2 hr in methionine-free Dulbecco's modified Eagle medium with 10% FBS. Then, Tran ^{35}S-label™, *E. coli* hydrolysate labeling reagent, containing ^{35}S-methionine (ICN Biomedicals, Irvine: specific activity >1000 Ci/mmol) was added to the medium to a final concentration of 10 μCi/ml and transferred to desired temperatures and incubated for additional 2 hr. Subsequently, the medium was removed and the cells were washed with PBS(−), harvested by a small rubber policeman. The cells were suspended in Laemmli's buffer [8], and boiled for 3 min.

The protein was analyzed on 10% polyacrylamide-SDS slab gels with 2.5% stucking gel using the discontinuous buffer system of Laemmli [8]. Protein samples with approximately the same ^{35}S counts (about 30,000 cpm) were used for analysis. Slab gels (5 cm×8.5 cm) were run at 15 mA for about 110 min, and stained with silver stain (2D-Silver Stain Kit 'DPC'; Daiichi Pure Chemical, Tokyo). The gels were dried and autoradiographed using Kodak X-Omat R5 film.

RESULTS

The cells dispersed from caudal fin of the goldfish and the remaining tissue pieces attached to the plastic substratum. Many cells migrated from tissue pieces and continued to proliferate both at 37°C and 27°C. After 18 days of incubation, GTF e-2 and GTF e-3 cells reached confluency. At that time the total cell numbers were 5×10^6

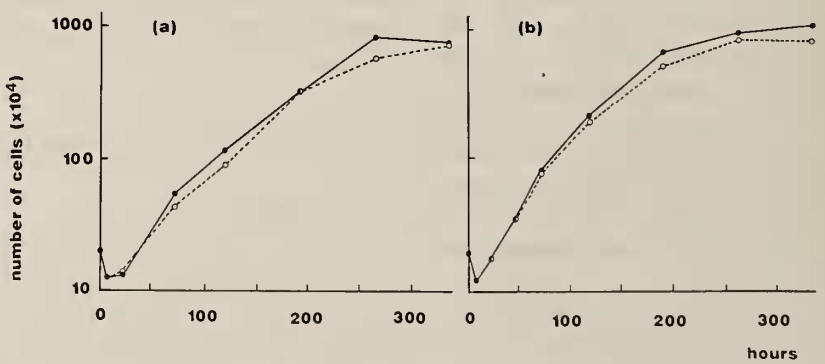

FIG. 1. Growth curve of GTF e-2 and e-3 cells at 27°C (a) and 37°C (b). The ordinate is the average number of cells recovered from a dish, and the abscissa is time in hours after inoculation. ●—●: GTF e-2, primary culture was started at 37°C. ○—○: GTF e-3, primary culture was started at 27°C.

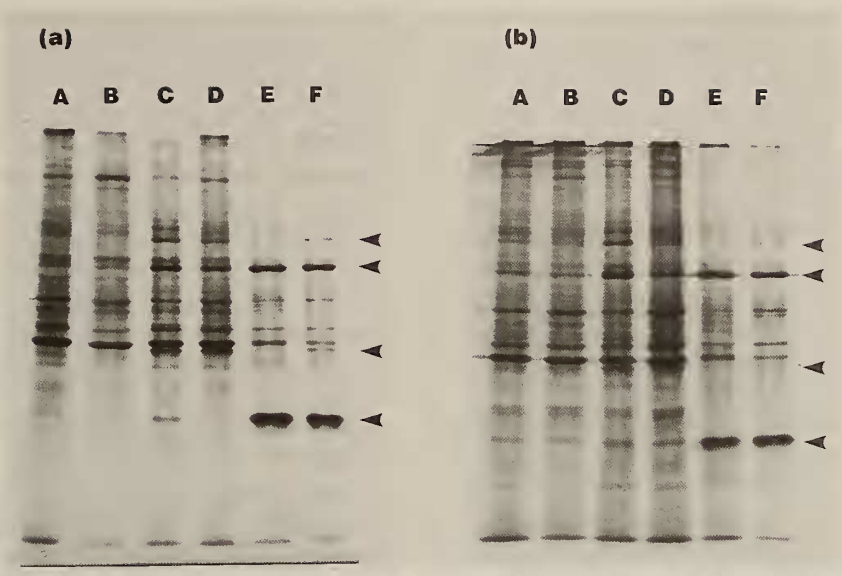

FIG. 2. The autoradiograph of heat shock proteins in (a) GTF e-3, and (b) GTF e-2. The temperature was shifted as shown below:
lane A: 27°C (12 hr)–27°C (labeled for 2 hr)
lane B: 37°C (12 hr)–27°C (labeled for 2 hr)
lane C: 27°C (12 hr)–37°C (labeled for 2 hr)
lane D: 37°C (12 hr)–37°C (labeled for 2 hr)
lane E: 27°C (12 hr)–40°C (labeled for 2 hr)
lane F: 37°C (12 hr)–40°C (labeled for 2 hr)
The cells were labeled with ^{35}S-methionine at the corresponding times. The arrows indicate hsp 90, hsp 70, hsp 42 and hsp 30 (from top to botoom), respectively.

for GTF e-2 and 4×10^5 for GTF e-3, and the morphology of the cells did not show any observable difference between the two cell strains. Both cell strains continued to grow without any sign of crisis of growth. This is one of the notable characteristics of cultured fish cells reported previously [4, 9, 10]. Figure 1 shows growth curves of GTF cells at early passages (PDN$<$11). The doubling time of GTF e-2 cells calculated from the slope of the initial straight line portion of the growth curve was 35 hr at 27°C, and 25 hr at 37°C. The doubling time for GTF e-3 cells was 36 hr at 27°C, and 25 hr at 37°C. Thus, the doubling time of cultured goldfish cells at early passages did not depend on the temperature at which the primary culture was started, but depended only on the incubation temperature.

Figure 2 shows the autoradiographs of newly synthesized proteins. The four major proteins were identified when the cells were transferred to higher incubation temperature. The molecular weights of hsps observed in the present study were 90, 70, 42 and 30-kD, and would correspond respectively to hsp 90, hsp 70, hsp 42 and hsp 30 of RBCF-1 cells [7]. The synthesis of these proteins was markedly increased when the GTF e-2 and GTF e-3 cells were incubated first at 27°C for 12 hr and then transferred to 37°C or after a long incubation at 37°C. At higher temperature (40°C) the hsp 70 and hsp 30 became dominant newly synthesized protein, and relative amount of hsp 90 and hsp 42 synthesis decreased in both cell strains.

DISCUSSION

It is generally accepted that cultured cells grow optimally when the incubation temperature is slightly higher than that preferred by the intact animal. For example, in case of cold-water fish like rainbow trout (*Salmo gairdnerii*), the cultured

cells grow most rapidly at temperature from 8 to 12°C [3]. As to goldfish cell lines, the optimal growth temperature reported has not been consistent. Rio *et al.* [4] reported 20°C for SJU-1 cell line, while 33°C was reported for CAF cell line by Etoh and Suyama [5]. Shima *et al.* established the RBCF-1 cell line which was initially cultured at 37°C and could grow at a wide range of temperatures from 20°C to 37°C (eurythermic growth) [6]. Recently, after a long term cultivation at 27°C or 37°C, clones of RBCF-1 line which could grow only at a narrow range of temperatures (stenothermic growth) were isolated [7].

In this study, we found that both GTF e-2 and e-3 cells which were derived from a goldfish tail fin, retained eurythermic growth properties at early passages (PDN<11), in spite of 10°C difference in primary culture. A probable cause for difference between eurythermic and stenothermic growth may be the difference in the length of their subcultivation time. SJU-1 cell line was at 110th passage after 39 months of subcultivation during which periods the cells were cultured at 20°C. RBCF-1 cells have been subcultured for more than 10 years. GTF cells were at only 2nd passage (the PDN is 3.2 for GTF e-2, and 1.3 for GTF e-3) after 4 days of subcultivation when they were used for the experiments. The intact goldfish as individuals can survive both at 27°C and 37°C, and this fact seems to correlate with the growth properties of GTF cells. So the cells *in vivo* may have the eurythermic growth properties. SJU-1 and RBCF-1 cells, which have been cultured for a long time at a constant temperature, may have lost their ability to grow eurythermically.

The molecular weights of major heat shock proteins synthesized by GTF cells were almost the same as those synthesized by cells of *Drosophila* [11, 12], mammals [13] and rainbow trout [14]. The hasp 90 of GTF cells may correspond to hsp 83 of *Drosophila* in the manner of response to the change of temperature; hsp 83 was not induced at a higher temperature (38°C) in *Drosophila* [11], and in GTF cells hsp 90 was not induced at 40°C.

The hsp 42 was induced in GTF cells when the cells were transferred from 27°C to 37°C, and also when they had been kept at 37°C. This response to temperature shift was similar to that of hsp 90. The cells of *Drosophila* do not synthesize hsp 42 [11, 12]. Rainbow trout cells (hsp 42) [14], chicken embryo fibroblasts (hsp 47) [15] as well as HeLa cells (hsp 43) [13] synthesize this class of hsp, all of which may correspond to hsp 42 of GTF cells. Therefore, hsp 42 may be the common heat shock protein in the cells of vertebrates so far examined.

The cells of *Drosophila* synthesize hsp 70 and hsp 26 which correspond to has 70 and hsp 30 of GTF cells. The hasps 70 and 26 are reported to be induced at a higher temperature than the temperature which induced hsp 90 [12]. This was also observed in GTF cells.

The relationships between the range of growth temperatures of cells and the temperature which can induce heat shock proteins in the cells have been reported for only a few species [11–14]. However, it may generally be said that hsps syntheses are induced when cells are transferred to a temperature which is a few degrees higher than their optimal growth temperature. Furthermore, the heat shock proteins may be synthesized as a consequence of environmental stress. In this study, we found that GTF cells do not follow this pattern, at 37°C hsps seem to be induced continuously, although the cells could grow actively at that temperature. This is quite different from RBCF-1 cells. When RBCF-1 cells were transferred from 26°C to 37°C, four major heat shock proteins were induced, but after 12 hr incubation synthesis of heat shock proteins decreased [7].

To summarize, in this study we found that cultured fish cells in the very early passages (PDN <3.2) can grow actively at both 27°C and 37°C. This eurythermic growth may reflect a growth characteristic of goldfish cells *in vivo*. It was also observed that in the cultured goldfish cells at early passages, four major hsps were induced at 37°C in spite of continued growth at this temperature for a long time. These results indicate that primary cultured goldfish cells are quite useful for investigating factors that determine the optimal growth temperature of cells, and the function of hsps in eurythermic animals.

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